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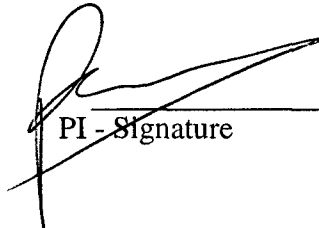
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FOREWORD

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TABLE OF CONTENTS

Front Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	
Body	
Key Research Accomplishments	5 - 6
Reportable outcomes	
Conclusions	
References	
Appendices	7

5. INTRODUCTION:

In 20-30% of breast tumors, ErbB2, a receptor tyrosine kinase (RTK) of the ErbB/EGFR family, is overexpressed (refs 1, 2, 3, 4) suggesting that molecules that downregulate the activity of this receptor kinase should act as efficient therapeutics in the management of breast cancers. Further, other members of the ErbB family, namely ErbB3 and ErbB4 have been implicated in the development of human breast cancers. Through our studies of the *Drosophila* homologue of ErbB RTK, we have discovered a transmembrane protein, named Kek1, that acts as a negative regulator of the EGFR. Kek1 (ref 5) is a member of a novel family of proteins and we have found that it is also a potent inhibitor of the EGFR in mammalian cells.

6. BODY:

In the previous application four specific aims were described to understand the regulatory mechanism by which Kek proteins inhibit the activity of members of the ErbB family of RTKs. We proposed to first determine precisely the molecular mechanism by which the *Drosophila* protein Kek1 inhibits the activity of the *Drosophila* DER receptor. Second, we proposed to determine whether Kek1 and its mammalian homologs inhibit the neoplastic state triggered by overexpression of the ErbB RTKs. Specifically, the goals were

1: We will define using the *Drosophila* system the molecular mechanism by which Kek1 inhibits DER activity.

2: We will search for both murine and human Kek1 homologs, examine their expression in mammary tissues and define their chromosomal locations.

3: We will determine whether Kek proteins can inhibit the activity of various mammalian ErbB RTKs in transformed cell lines.

4: We will test in transgenic mice whether Kek proteins can inhibit the occurrence of ErbB-induced mammary carcinomas.

In the first year of the award, we have made significant progress towards the initial goals. Namely, we have completed aims 1 and 3. Three manuscripts, one published in *Cell*, and two additional ones which are almost completed, have resulted from this work. We have found that the *Drosophila* transmembrane molecule kek1 acts in a negative feed back loop to modulate the activity of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase. kek1 is expressed in response to activation of the Gurken/EGFR signaling pathway during oogenesis. While loss of kek1 activity is associated with a phenotype reminiscent of increased Grk/EGFR signaling, ectopic overexpression of kek1 mimics the complete loss of EGFR activity. We found that the extracellular domain of Kek1 physically associates with the EGFR providing the basis for this inhibitory mechanism. Interestingly, we found that Kek1 is also a potent inhibitor of the EGFR in mammalian cells. Kek1 binds the EGFR and related proteins ErbB2, ErbB3 and ErbB4. Kek1 interferes with EGF mediated receptor tyrosine phosphorylation and activation of the downstream signaling molecules PI3-kinase and Erk/MAP kinases. Kek1 can also inhibit transformation in mouse mammary tumor cells with deregulated expression of receptors and ligands of the ErbB family.

In the second year of funding of the grant, we plan to initiate the projects relevant to aims 2 and 4 of the grant application.

7. KEY RESEARCH ACCOMPLISHMENTS:

We have shown that:

1. Kek1 inhibits the activity of the *Drosophila* EGFR

2. the extracellular domain of Kek1 physically associates with the EGFR providing the basis for this inhibitory mechanism.

3. Kek1 is also a potent inhibitor of the EGFR in mammalian cells. First, Kek1 binds the EGFR and related proteins ErbB2, ErbB3 and ErbB4.

4. Kek1 interferes with EGF mediated receptor tyrosine phosphorylation and activation of the downstream signaling molecules PI3-kinase and Erk/MAP kinases.

5. Kek1 can inhibit transformation in mouse mammary tumor cells with deregulated expression of receptors and ligands of the ErbB family.

8. REPORTABLE OUTCOMES:

Published Manuscripts:

Ghiglione, C., Carraway, K.L., Amundadottir, L.T., Boswell, R.E., Perrimon, N.*, and Duffy, J.B.* (1999). The transmembrane molecule Kekk1 acts in a feed back loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis.* Co-senior and corresponding authors. Cell 96. 847-856.

Manuscripts in preparation:

Amundadottir, L.T., Ghiglione, C., Perrimon, N. and Carraway, K.L. Drosophila Kek1 antagonizes the activity of mammalian EGFR/ ErbB receptor tyrosine kinases. In Preparation.

Ghiglione, C., Carraway, K.L., and Perrimon, N. The transmembrane molecule Kekk1 blocks the activity of the Drosophila EGF receptor at multiple times during development. In Preparation.

Patent:

Kekk1 genes and gene products, filed.

9. CONCLUSIONS:

By examining how Kekk1 proteins are able to selectively block the activity of the ErbB family members, we may be able to design a novel strategy to control the development of breast cancers associated with misregulation of ErbB genes. Our studies on Kekk1 proteins may have two direct applications to breast cancer research. First, it may lead to the design of small molecule screens to identify compounds that mimic the effect of Kekk1. Identification of such compounds may act as efficient therapeutics against ErbB-induced cancers. Second, because we expect that human mutations in kek1 homologs will be associated with upregulation of ErbB activity, it is possible that kek1 genes will behave as tumor suppressors. Ultimately, diagnosis of kek1 mutations could be performed to determine the predisposition of individuals to breast cancers.

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11. APPENDICES:

Ghiglione, C., Carraway, K.L., Amundadottir, L.T., Boswell, R.E., Perrimon, N.*, and Duffy, J.B.*

(1999). The transmembrane molecule Kekkon 1 acts in a feed back loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis.* Co-senior and corresponding authors. Cell 96. 847-856.

The Transmembrane Molecule Kekk1 Acts in a Feedback Loop to Negatively Regulate the Activity of the *Drosophila* EGF Receptor during Oogenesis

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Summary

We have identified the *Drosophila* transmembrane molecule *kek1* (*kek1*) as an inhibitor of the epidermal growth factor receptor (EGFR) and demonstrate that it acts in a negative feedback loop to modulate the activity of the EGFR tyrosine kinase. During oogenesis, *kek1* is expressed in response to the Gurken/EGFR signaling pathway, and loss of *kek1* activity is associated with an increase in EGFR signaling. Consistent with our loss-of-function studies, we demonstrate that ectopic overexpression of *kek1* mimics a loss of EGFR activity. We show that the extracellular and transmembrane domains of Kek1 can inhibit and physically associate with the EGFR, suggesting potential models for this inhibitory mechanism.

Introduction

The biological processes regulated by receptor tyrosine kinase (RTK) signaling pathways are diverse and include the regulation of cell growth, differentiation, migration, viability, and maintenance of homeostasis (see review by Ullrich and Schlessinger, 1990). In the past few years, a great deal has been learned about the mechanisms by which RTKs transduce signals and specify particular cellular fates. In particular, most RTKs have been shown to regulate gene expression through the Ras/Raf/MEK/MAPK cassette (see Li and Perrimon, 1997). Although the pathways that transduce signals from RTKs to the nucleus have been well characterized, less is known about the mechanisms involved in imparting specificity. More recently, however, it has been shown that modulation of RTK activities is a critical aspect of their biological

roles, since quantitative variations in their level of activations can lead to very different cellular outcomes. For example, studies in mammalian PC12 cells have illustrated that prolonged MAPK activation and nuclear translocation triggers differentiation, whereas transient activation leads to proliferation (see Li and Perrimon, 1997). Similarly, in the *Drosophila* embryos, patterning of the terminal region is determined precisely by the strength of the signal generated by the Torso RTK (Greenwood and Struhl, 1997). Thus, precise regulation of the quantitative output of a signal generated by an RTK is critical to the control of growth and differentiation.

While many positive regulators/effectors of RTK signaling activity have been characterized, only a few inhibitory molecules have been identified (Freeman et al., 1992; Kharitonov et al., 1997). In addition to these inhibitory molecules, a number of inhibitory mechanisms have been identified. These include internalization of ligand–receptor complexes, receptor desensitization as a result of hyperphosphorylation of the receptor, inhibitory regulation by cytoplasmic and transmembrane protein phosphatases, and targeting to the proteolytic pathway of specific signal transducers (Ullrich and Schlessinger, 1990; Sturtevant et al., 1994; van der Geer et al., 1994; Kokel et al., 1998). By integrating particular stimulatory and inhibitory mechanisms in cells, RTK activities can be quantitatively regulated to direct distinct cellular outcomes.

The *Drosophila* EGFR is required for a multitude of developmental processes throughout the life cycle (reviews by Ray and Schupbach, 1996; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). For example, activation of the EGFR pathway in follicle cells during oogenesis establishes both anteroposterior and dorsoventral fates. During embryogenesis, the EGFR is required for the establishment of ventral cell fates, determination of segmental identity, maintenance of amnioserosa and ventral neuroectodermal cells, germband retraction, cell fate specification in the central nervous system, and production of cuticle. During larval development, the EGFR plays a role in cell proliferation, vein formation, and eye development. These multiple tissue-specific activities are regulated in part by different ligands that contain an EGF repeat similar to that of transforming growth factor α (TGF α), a known ligand of the vertebrate EGFR. To date, three such ligands have been isolated in *Drosophila*: Spitz (Spi; Rutledge et al., 1992; Schweitzer et al., 1995a), Gurken (Grk; Neuman-Silberberg and Schupbach, 1993), and Vein (Vn; Schnepf et al., 1996). In contrast to these stimulatory molecules, Argos (Aos), a secreted protein with an atypical EGF motif, has been shown to act as an antagonist of EGFR activity (Schweitzer et al., 1995b). In vitro studies have revealed that Aos can interfere with EGFR activation in a manner that is both saturable and competitive, and it has been proposed that Aos acts as a direct inhibitor of Spi by binding directly to the EGFR (Schweitzer et al., 1995b). Remarkably, the expression of *aos* is dependent upon EGFR activation (Golembo et al., 1996b), thus demonstrating

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that the EGFR regulates the expression of its own negative regulator.

During late oogenesis, EGFR activity is regulated by all of these mechanisms, and its precise activity is critical in specifying the proper pattern of the chorion and embryo (review by Ray and Schupbach, 1996). Thus, function of the EGFR during oogenesis provides an excellent paradigm to characterize the quantitative regulation of the EGFR and determine if additional components of this pathway exist. In response to Grk signaling from the oocyte, activation of the EGFR in follicle cells follows a dynamic pattern. In early egg chambers, Grk first activates the EGFR pathway in posterior follicle cells. Subsequently, around stage 7, *grk* transcripts become localized to the anterodorsal corner of the oocyte and signal to adjacent follicle cells to define their dorsal fates. Activated EGFR molecules regulate transcription through the Ras/Raf/MEK/MAPK cassette in follicle cells (Brand and Perrimon, 1994; Hsu and Perrimon, 1994; Schnorr and Berg, 1996). In a screen to identify downstream genes regulated by the EGFR in follicle cells, we have identified the gene *kekkon 1* (*kek1*), which encodes a single pass transmembrane protein with features of a cell adhesion molecule with leucine-rich repeats (LRR) and immunoglobulin (Ig) motifs (Musacchio and Perrimon, 1996), as a target gene. We demonstrate that endogenous *kek1* acts in an inhibitory manner: loss of *kek1* activity results in increased EGFR signaling. We also demonstrate that overexpression of *kek1* blocks the activity of the EGFR. This inhibition involves a physical association between the extracellular and transmembrane domains of Kek1 with the EGFR. This establishes Kek1 as a bona fide inhibitor of the EGFR that acts in a negative feedback loop to modulate the activity of this RTK signaling pathway.

Results

kek1 Is Regulated by the Grk/EGFR Pathway in Follicle Cells

To characterize target genes of the EGFR signaling pathway, we screened a collection of enhancer trap lines for expression patterns in follicle cells. We found one line, 15A6, which was expressed in a pattern consistent with it being a regulatory target of the EGFR pathway, since it undergoes a transition during stages 8–10 when it relocates from a posterior to a dorsal–anterior gradient (Figures 1A–1C). 15A6 corresponds to an insertion in the *kek1* gene (Musacchio and Perrimon, 1996), and we refer to it as *kek-lacZ* in the text. We found that *kek-lacZ* expression reflects accurately the expression of *kek1* both in wild-type (WT) and in mutant backgrounds (data not shown).

To establish a regulatory link between the transcriptional regulation of *kek1* in the follicle cell epithelium and the Grk/EGFR signaling pathway, we examined *kek-lacZ* expression in mutants for *grk*, *egfr*, and the kinase *Draf* that is both necessary and sufficient for EGFR signaling in follicle cells (Brand and Perrimon, 1994). In each case, the dorsal–anterior gradient of *kek-lacZ* expression was abolished or severely diminished (Figures 1D and 1E; see also Brand and Perrimon, 1994; Queenan

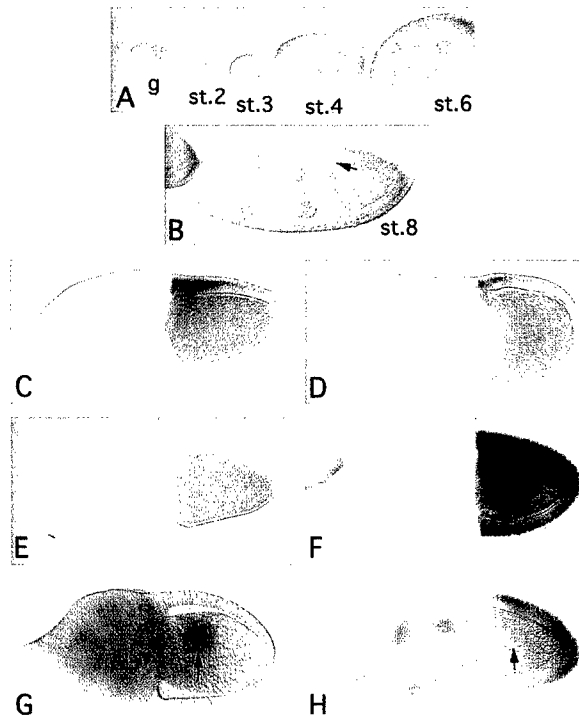


Figure 1. *kek1* Is a Transcriptional Target of the EGFR Pathway

(A–C) Expression of *kek1* in the follicle cells revealed by the *kek-lacZ* enhancer trap insertion. *kek-lacZ* expression can first be detected in region 2 of the germarium (A). Expression continues to be detected at the poles of stage 1–6 egg chambers. Following the dorsal–anterior migration of the oocyte nucleus, *kek-lacZ* expression undergoes a similar shift to a dorsal–anterior region of the follicular epithelium in stage 8 and 9 egg chambers (B). The position of the oocyte nucleus is marked by the arrow. A lateral view of a stage 10 egg chamber showing the dorsal–anterior graded expression of *kek-lacZ* (C).

(D–F) *kek-lacZ* expression is under the control of the EGFR signaling pathway. In females homozygous for the hypomorphic *Draf* mutation, *Draf^{HM7}* (Melnick et al., 1993) *kek-lacZ* expression is severely reduced (D). Expression of *kek-lacZ* is completely abolished in ovaries of females homozygous for *grk^{W38}* (E) or mutations in the *egfr* associated with female sterility (data not shown). Ectopic activation of the EGFR receptor pathway using the gain-of-function *Draf* gene *hsDraf^{poF22}* (F) leads to uniform *kek-lacZ* expression. Induction of WT *Draf* during oogenesis had no effect on the expression of *kek-lacZ* (not shown).

(G and H) The position of the oocyte nucleus defines the spatial domain of *kek1* expression. In response to colchicine treatment, the oocyte nucleus and its associated *grk* mRNAs become misplaced during oogenesis (G and H). In these egg chambers, the expression of *kek-lacZ* can be detected posteriorly as well as ventrally. In (G) and (H), the germline nuclei are marked with the enhancer trap line *es(3)79* to allow easy detection of the oocyte nucleus (indicated by the arrows).

(C–H) are stage 10 egg chambers oriented with dorsal up. Anterior is to the left in all panels.

et al., 1997; Sapir et al., 1998), revealing that *kek-lacZ* expression is transcriptionally regulated by the Grk/EGFR/*Draf* pathway. Expression of an activated form of *Draf* resulted in the ubiquitous expression of *kek-lacZ* within the follicular epithelium (Figure 1F).

To further analyze the role of the Grk/EGFR pathway in the transcriptional control of *kek-lacZ*, we mislocalized the subcellular localization of *grk* mRNAs by disrupting the oocyte cytoskeletal network (Figure 1G).

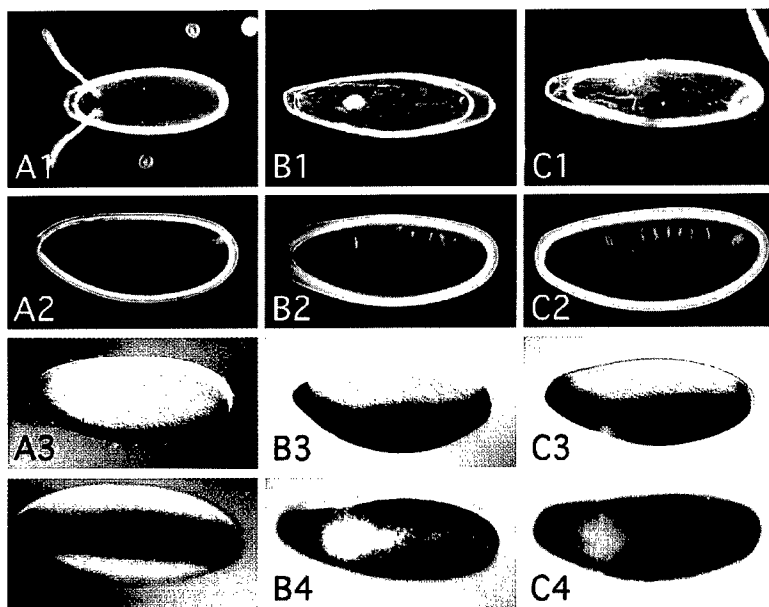


Figure 2. Overexpression of *kek1* during Oogenesis Blocks the Activity of the EGFR

Phenotypes of eggs and embryos derived from WT (A), *T155; UAS-kek1* (B), and *T155; UAS-egfrDN* (C) females. The eggshell is secreted by the follicle cells during oogenesis. Specialized groups of follicle cells on the dorsal side of the egg chamber form the two dorsal respiratory appendages. Note that the chorionic filaments, localized anteriorly in the WT, are fused, do not elongate, and are positioned more posteriorly when either *kek1* or the *egfrDN* is expressed uniformly in follicle cells using the Gal4 driver *T155*. The embryonic cuticles that develop from these eggs are strongly ventralized (cf. Figures B2 and C2 with the WT in Figure A2), as observed in embryos derived from *grk* homozygous mothers (Roth and Schupbach, 1994). This ventralization phenotype can be readily detected using the *Twi* marker for ventral cell fates. In WT embryos (A3 and A4), *Twi* is expressed in the ventral-most region of the embryo. The *Twi* expression domain is enlarged when either *kek1* or the *egfrDN* is misexpressed (B3, B4, C3, C4).

In all panels, anterior is to the left. The chorions shown in (A1), (B1), and (C1) are dorsal views. The embryos in (A2), (B2), (C2), (A3), (B3), (C3), and (A4), (B4), and (C4) are lateral and ventral views, respectively.

Treatment of the oocyte with colchicine, an inhibitor of microtubule polymerization, leads to mislocalization of the oocyte nucleus and its associated *grk* mRNAs, which correlated with ectopic *kek-lacZ* expression (Figure 1H) as well as *kek1* mRNA expression (not shown). Altogether, these results demonstrate that Grk is not only necessary but sufficient to regulate the spatial expression of *kek1*.

Overexpression of *kek1* during Oogenesis Antagonizes EGFR Activity

Previous analysis has revealed that in the complete absence of *kek1* gene activity, flies are viable, fertile, and do not exhibit any overt morphological defects (Musacchio and Perrimon, 1996). *Kek1* has the features of cell adhesion molecules (CAMs), and loss-of-function mutations of many CAMs have subtle mutant phenotypes. However, when ectopically overexpressed, some CAM molecules can generate striking mutant phenotypes that are revealing of their functions (Speicher et al., 1998). Thus, to gain insights into the possible function of *kek1* during oogenesis, we tested the effect of overexpressing *kek1* in the follicle cells using the GAL4/UAS system (Brand and Perrimon, 1993).

When *kek1* is expressed under the control of the GAL4 line *T155*, which drives expression all over the follicle cell epithelium beginning at stage 9 (Harrison et al., 1995; Queenan et al., 1997), 100% of the resulting eggs are longer than the WT eggs and have a reduction or a complete loss of dorsal appendages (cf. Figure 2B1 with the WT in Figure 2A1). This loss of dorsal appendage material is due to a ventralization of the eggshell, a phenotype associated with *egfr*, or *grk* loss-of-function mutations (Schupbach, 1987; Price et al., 1989). A similar ventralized eggshell phenotype was obtained when a dominant-negative form of the EGFR, *EGFRDN*, was expressed under the control of *T155* (Figure 2C1).

In addition to its role in establishing the dorsal characteristics of the eggshell, the Grk/EGFR pathway controls embryonic dorsoventral patterning by restricting a ventralizing signal (Neuman-Silberberg and Schupbach, 1994; Morisato and Anderson, 1995). As a result, loss of EGFR function leads to ventralized embryos. Embryonic cuticles derived from *T155; UAS-kek1* females showed a ventralized cuticle phenotype (Figure 2B2). To analyze the extent of this ventralization, we stained embryos for Twist (*Twi*) RNA and protein that label a ventral domain ten cells wide (Thisse et al., 1988). Overexpression of *kek1* in follicle cells results in an expansion of the *Twi* expression domain (Figures 2B3 and 2B4). Similarly, embryos derived from *T155; UAS-egfrDN* females are also strongly ventralized (Figures 2C2–2C4).

Altogether, our results indicate that overexpression of *kek1* in follicle cells ventralizes both the eggshell and the embryo by most likely interfering with the activity of the Grk/EGFR signaling pathway.

Kek1 Inhibits the Activity of the Ras/Raf/MEK/MAPK Cassette

To determine whether *Kek1* blocks the signaling activity of the EGFR, we tested whether ectopic expression of *kek1* blocks the transcriptional activation of target genes regulated by the EGFR/Ras/Raf/MEK/MAPK pathway in follicle cells. Since *kek1* is a target of this pathway, we used *kek-lacZ* as a reporter for this experiment. Overexpression of *kek1* using the Gal4 driver *T155* was associated with a strong but not complete reduction of *kek-lacZ* expression (cf. Figure 3B to WT in Figure 3A). Using a stronger Gal4 line, *CY2* (Queenan et al., 1997), a complete disappearance of *kek-lacZ* expression was observed (Figure 3C). These data are consistent with the model that *Kek1* downregulates the activity of the Ras/Raf/MEK/MAPK pathway. Consistent with this, epistasis experiments indicate that the inhibitory effect of *Kek1*



Figure 3. Kek1 Inhibits the Activity of the Ras/Raf/MEK/MAPK Cascade

Expression of *kek-lacZ* in WT (A) and following ectopic overexpression of *kek1* in the follicular cell epithelium using either the T155 (B) or CY2 Gal4 (C) drivers. Only the most dorsal anterior patch of follicle cells at stage 10 are still expressing *kek-lacZ* in (B), while the expression of the reporter gene is completely absent in (C). The genotypes of the egg chambers shown in (B) and (C) are *UAS-kek1/kek-lacZ; T155* and *UAS-kek1; kek-lacZ/CY2*, respectively. All panels are dorsal views of stage 10 egg chambers stained with X-Gal.

is overridden by the constitutive activation of the EGFR or Draf (Figure 4). In addition, we tested the interaction between the transmembrane protein Rhomboid (Rho) and Kek1. Rho has been proposed to play a role in the activation of the EGFR (Schweitzer et al., 1995a; Golembo et al., 1996a), and overexpression of Rho leads to dorsalized eggshells (Ruohola-Baker et al., 1993). We find that overexpression of *rho* does not override the

ventralization phenotype of *UAS-kek1* (Figure 4), indicating that Kek1 can block the effect of Rho on EGFR activation. Altogether, these results place Kek1 upstream of the EGFR and downstream of Rho.

The Extracellular Domain of Kek1 Is Critical for the Inhibitory Effect

To test whether the extracellular domain of Kek1, which contains five LRR and one Ig motif (Musacchio and Perrimon, 1996), is required for the inhibition of the EGFR activity by Kek1, we generated transgenic lines that contain either *UAS-kek1^{extra}* or *UAS-kek1^{intra}*.

No phenotype were observed by overexpressing *kek1^{intra}* (data not shown) in the follicle cells using the T155 or CY2 GAL4 lines. On the other hand, overexpression of *kek1^{extra}* using the same drivers led to eggs with reduced dorsal appendage materials (Figure 5A) and ventralized embryos (Figures 5B and 5C). These observations indicate that the extracellular domain of Kek1 is sufficient to inhibit the activity of the EGFR.

Although the phenotypes obtained following *kek1^{extra}* overexpression are very similar to those obtained with full-length *kek1*, we note that these phenotypes are weaker. One possibility is that *Kek1^{extra}* is expressed at a lower level than the WT protein. Weaker effects were observed with 12 independent transgenic lines, as well as when multiple copies of the transgenes were added. This suggests that *Kek1^{extra}* is less stable than the full-length Kek1 molecule or that the cytoplasmic domain of Kek1 participates to some extent in the inhibition.

Finally, we tested whether the inhibition of the EGFR activity by *kek1^{extra}* requires the presence of the endogenous Kek1 protein. When *kek1^{extra}* was overexpressed in *kek1* mutant females, the eggs were ventralized, which excludes a model whereby *kek1^{extra}* blocks the EGFR by forming a heterodimer with the endogenous Kek1 protein (data not shown).



Figure 4. Genetic Epistasis between Kek1, Rho, and Constitutively Activated Forms of Either D-Raf or the EGFR (λ Top)

(A) Chorion phenotypes. (1) Eggs derived from *UAS-Kek1; UAS-Rho/T155* females are strongly ventralized. (2) Eggs derived from *UAS-Kek1; UAS- λ top/T155* females are dorsalized and are similar to those laid by *UAS- λ top/T155* females. Dorsalization is clearly visible by the extraappendage materials deposited around the entire egg circumference. (B) Summary table of the various genotypes analyzed.

Genotype	Chorion phenotype		
	WT	Ventralized	Dorsalized
<i>UAS-Kek1^{3LX/+}; T155/+</i>	1	770	
<i>UAS-Rho1^{10/+}; T155/+</i>			633
<i>UAS-Kek1^{3LX/+}; UAS-Rho1^{10/+}; T155/+</i>		652	
<i>UAS-Kek1^{3LX/UAS-λtop⁴²; T155/+}</i>	4		742
<i>UAS-λtop⁴²; T155/+</i>			875
<i>UAS-Kek1^{3LX/+}; UAS-DRaf^{R29}/T155</i>	4	2	192
<i>UAS-Kek1^{3LX/+}; UAS-DRaf^{R29}/T155</i>			98

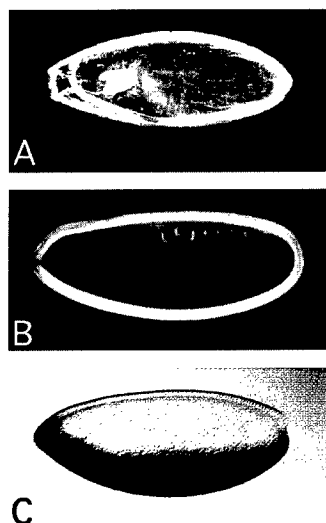


Figure 5. The Extracellular Domain of Kek1 Is Sufficient to Inhibit the Activity of the EGFR

Eggs derived from CY2; *UAS-kek1^{extra}* females that express *kek1^{extra}* in follicle cells using the Gal4 line CY2 exhibit a ventralized eggshell phenotype (A). Consistently, the embryos that develop from these eggs show a ventralized cuticle phenotype (B) and a lateral expansion of Twi expression (C). (A) is a dorsal view, and (B) and (C) are lateral views.

Kek1 Is a Negative Regulator of the Grk/EGFR Signaling Pathway

Because of the severe inhibition of EGFR activity by Kek1 revealed by the overexpression experiment, we decided to reexamine the effect of loss of *kek1* function during oogenesis. We reasoned that a subtle egg morphology phenotype may have been missed by simply using fertility as an assay (Musacchio and Perrimon, 1996). Indeed, we found that the spacing between the dorsal appendages of eggs derived from *kek1* mutant females was increased when compared to WT. Further, these eggs were also mildly shorter and rounder (Figure 6A1), a phenotype consistent with a hyperactivation of the Grk/EGFR pathway (Neuman-Silberberg and Schupbach, 1994). These features do not interfere with hatching rates and patterning that is consistent with the normal Twi expression found in *kek1* mutant embryos ($n = 142$). Interestingly, when *kek1* mutant flies were raised at 29°C, 5% ($n = 257$) of the embryos derived from *kek1* mutant females showed a mild reduction in Twi expression (Figures 6A2 and 6A3). We conclude that loss of *kek1* activity during oogenesis leads to mildly dorsalized eggs.

To substantiate the functional relationship between Kek1 and the Grk/EGFR signaling pathway during oogenesis, we examined the effects of a loss of Kek1 activity in two different genetic backgrounds. First we tested if an increase in the level of Grk molecules can generate a stronger phenotype in the absence of *kek1*. Previously, Neuman-Silberberg and Schupbach (1994) showed that females carrying four copies of a transgene expressing *grk* (*P[grk]*), in addition to the two endogenous copies, lay a significant fraction of partially or severely dorsalized eggs. In contrast, females that carry

only one copy of *P[grk]* lay WT eggs (Neuman-Silberberg and Schupbach, 1994; this study, data not shown). We found that eggs laid by females carrying one copy of *P[grk]* in the absence of *kek1* are significantly dorsalized (Figure 6B1): the spacing between the dorsal appendages is increased and the eggs are rounder and smaller. Further, Twi expression that is normally expressed in embryos laid by females carrying one copy of *P[grk]* ($n = 168$; data not shown) is repressed ventrally in 15% ($n = 181$) of the embryos derived from *kek1* mutant females that carry a single copy of *P[grk]*. When raised at 29°C, 22% ($n = 348$) of the embryos derived from eggs laid by females carrying one copy of *P[grk]* in the absence of *kek1* showed a dorsalized phenotype readily detectable by aberrant Twi expression. We note that there is a variability in the reduction of the Twi domain, ranging from embryos in which Twi-expressing cells are missing in the middle regions of the embryo to the most severe cases in which only the poles express the Twi protein (Figures 6B2 and 6B3).

Second, we reasoned that if loss of Kek1 activity leads to a hyperactivation of EGFR activity, then loss of Kek1 activity should rescue a decrease in EGFR activity. We tested this hypothesis by generating flies that simultaneously lacked Kek1 activity and had reduced activity of the EGFR. Consistent with our hypothesis, loss of Kek1 activity resulted in partial suppression of the EGFR phenotype (Figures 6C1–6C3). Eggs laid by females mutant for EGFR alone have a ventralized phenotype (Schupbach, 1987). They exhibit either no or only a single dorsal appendage, and the eggs are significantly longer than WT (100%, $n = 852$; Figures 6C1 and 6C2). In contrast, the majority of eggs (95.5%, $n = 1103$) laid by the *kek1⁻ egfr⁻* double mutant females have two dorsal appendages and their length is shorter, consistent with a reversion to a more WT phenotype (Figure 6C3).

Kek1 Physically Associates with the EGFR

Having demonstrated that the *in vivo* role of *kek1* is consistent with the results obtained by overexpression, we wanted to gain insight into the mechanism of inhibition of the EGFR by Kek1. To do so, we tested whether EGFR and Kek1 physically associate. Following coexpression of a Myc-tagged version of Kek1 and the EGFR or the *Drosophila* Torso RTK in Sf9 cells, Kek1 was immunoprecipitated from the cell lysates using an anti-Myc antibody. Coprecipitation of the EGFR was observed by probing the resulting blot with the anti-EGFR antibody (Figure 7A), suggesting that Kek1 associates physically with the EGFR and that this interaction is responsible for the inhibitory effect. When both Kek1 and Torso were coexpressed in Sf9 cells, no Torso was coprecipitated by Kek1 (Figure 7A), suggesting a selectivity of Kek1 for binding to the EGFR.

Because the extracellular and transmembrane portions of Kek1 are essential for the inhibitory effect, we examined whether these portions of Kek1 are able to bind to the EGFR. As shown in Figure 7B, the extracellular domain, but not the intracellular domain, of Kek1 can bind to the EGFR. Thus, we propose that the inhibitory effect of the EGFR by Kek1 is mediated through direct association of the extracellular and transmembrane domains of Kek1 with the EGFR.

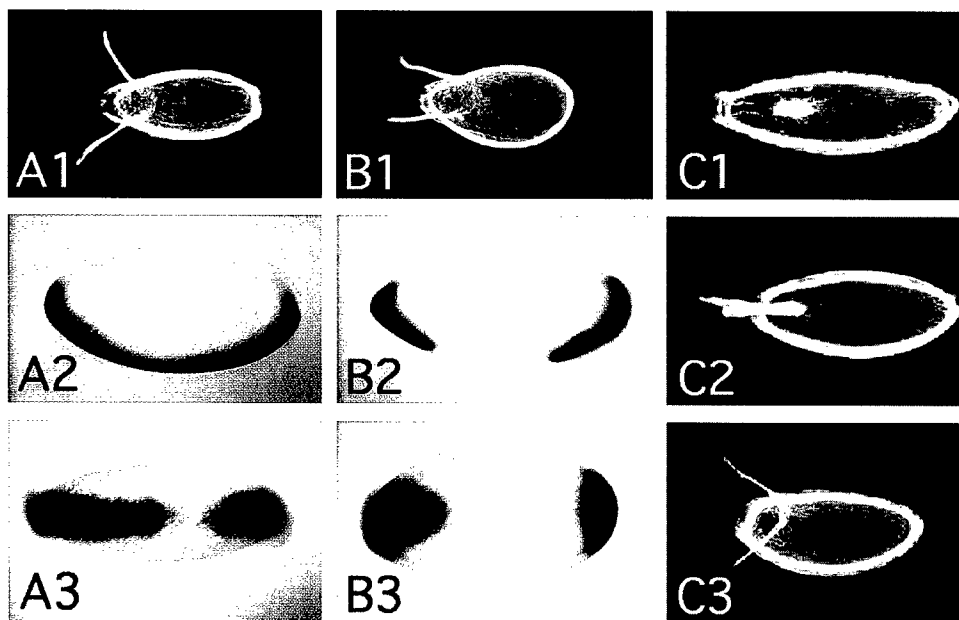


Figure 6. Phenotypes Associated with Loss of *kek1* Function during Oogenesis

(A1–A3) At 25°C, eggs derived from *kek1* mutant females showed a weak dorsalization phenotype that does not interfere with fertility. This dorsalization phenotype is evident by the more lateral position of the dorsal appendages (cf. Figure 6A1 with a WT eggshell shown in Figure 2A1). By increasing the temperature, we observed a weak enhancement of both the eggshell and the embryonic phenotypes readily detectable by aberrant *Twi* expression (Figures 6A2 and 6A3).

(B1–B3) The dorsalization phenotype associated with loss of *kek1* function is enhanced by an increase in the level of Grk ligand. The eggs derived from *kek1* mutant females that contain an extra copy of *grk*, provided by a *P[grk]* transgene, showed an enhancement of the dorsalized eggshell phenotype (cf. Figure 6B1 with Figure 6A1), and the embryos derived from these eggs showed a stronger dorsalized phenotype (cf. Figures 6B2 and 6B3 with Figures 6A2 and 6A3). Orientations are the same as in Figure 2.

(C1–C3) Suppression of the egg phenotype associated with *egfr* mutations by *kek1*. Chorions derived from *egfr*^{QY1} or *kek1*^{QY1} females are shown. (C1) *egfr*^{QY1}/*egfr*^{2E07}. (C2) *egfr*^{QY1}/*kek1*^{RM2} *egfr*^{2E07}. (C3) *kek1*^{QY1}/*kek1*^{RM2} *egfr*^{2E07}. In addition to the recovery of the two dorsal appendages, the length of the chorion has also become similar in length to WT.

Discussion

We have identified the transmembrane protein Kek1 as a molecule that acts in a negative feedback loop to modulate the activity of the *Drosophila* EGFR tyrosine kinase during oogenesis. We provide loss-of-function, ectopic overexpression, and biochemical evidence to support this. First, eggs laid by females that lack *kek1* gene activity are weakly dorsalized, and loss of *kek1* gene activity can suppress *egfr* mutations and potentiate Grk signaling. Second, ectopic overexpression of Kek1 in follicle cells leads to severe ventralization identical to effects seen in EGFR mutants, providing further support of Kek1 as a negative regulator of the EGFR pathway. Finally, we provide biochemical evidence that the inhibition of EGFR activity by Kek1 involves the direct association between the extracellular and transmembrane domains of Kek1 and the EGFR.

Interestingly, *kek1* is expressed in the eye and wing imaginal discs in a pattern that is highly suggestive of induction by the EGFR (Musacchio and Perrimon, 1996). Although no obvious phenotypes have been described in these tissues in *kek1* mutants, we found that overexpression of *kek1* in these tissues also generate phenotypes reminiscent to loss of EGFR activity (not shown). This suggests that the negative regulation of the EGFR by Kek1 is not only restricted to oogenesis.

The Function of Kek1 in Patterning of the Follicle Cell Epithelium

During oogenesis, Grk derived from the oocyte activates in a paracrine fashion the EGFR in dorsal follicle cells. Recent work from Wasserman and Freeman (1998) has shown that this paracrine signaling leads to the activation of a second phase of signaling, whereby the EGFR activity is amplified among follicle cells themselves. During this second phase, the EGFR activates a number of target genes that include both positive (*rho* and *vn*) and negative (*aos*) regulators of the pathway. Activation of Rho in follicle cells presumably leads to the activation of the Spi EGFR ligand, while activation of Aos within the peak of EGFR activity at the dorsal anterior leads to repression of EGFR, effectively splitting the initial peak of EGFR activity into two. This splitting of EGFR activity eventually defines the domains where the dorsal appendages will form.

Our identification of Kek1, together with the studies on Aos, indicates that there are at least two different negative regulators of EGFR activity in follicle cells. However, the regulation and function of Kek1 is distinct from those of Aos. *aos* is expressed only in response to high levels of EGFR activity, while *kek1* is expressed in a graded fashion. Further, loss-of-function phenotypes of *kek1* and *aos* in follicle cells are different. In the absence of *kek1* activity, the spacing between the dorsal appendages is increased, while in the absence

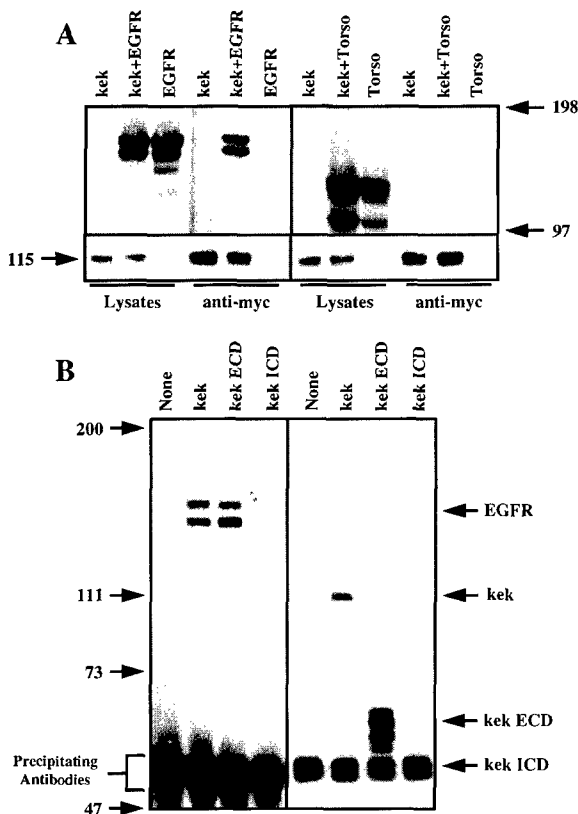


Figure 7. Association of Kek1 with the *Drosophila* EGFR

(A) Lysates from Sf9 insect cells expressing the *Drosophila* EGFR or Torso, or Kek1-Myc (kek) alone, or coexpressing Kek1-Myc with each of the RTKs were immunoprecipitated with anti-Myc antibodies. Precipitates were immunoblotted with anti-EGFR (upper left panel) or anti-Torso (upper right panel) and reprobed with anti-Myc (lower panels). In Sf9 cells, the EGFR migrates at two prominent forms. The lower EGFR band probably represents an underglycosylated form of the receptor. We noted that at higher Kek1 expression levels binding to Torso was also observed (not shown), perhaps reflecting some degree of stickiness of the Kek1 protein.

(B) Sf9 cells were infected with baculovirus encoding the *Drosophila* EGFR and coinfected with nothing (None) or viruses encoding Myc-tagged versions of full-length Kek1 (kek), a form encompassing the transmembrane and the extracellular domains of Kek1 (kek ECD), and a form encoding the intracellular domain of Kek1 fused to a v-Src myristylation site (kek ICD). Anti-Myc immunoprecipitates were blotted with anti-EGFR (left panel) and then reprobed with anti-Myc (right panel). Note that the 55 kDa Kek1 intracellular domain is hidden in the Myc immunoprecipitate by the heavy chain of the precipitating antibody, but it has been detected in the lysates.

of aos, the appendages are fused dorsally. Aos has been proposed to split the initial peak of EGFR activity into two (Wasserman and Freeman, 1998). We propose that the function of Kek1 is to restrict the lateral spreading of EGFR activation by Spi. Thus, in the absence of *kek1* activity, Rho/Spi activation could spread more laterally, explaining the enhancement of the spacing between the two dorsal appendages.

Mechanism of Inhibition of the EGFR by Kek1

Our analysis demonstrates that Kek1 acts as a potent negative regulator of EGFR activity when overexpressed.

Further, we have shown that the extracellular and transmembrane domains of Kek1 are sufficient for this inhibition. The extracellular domain of Kek1 contains one Ig-like domain and five LRRs, both of which can mediate protein-protein interactions (Musacchio and Perrimon, 1996). A number of mechanisms can underlie the mechanism by which this extracellular domain acts as an inhibitor. For example, the Kek1 extracellular domain could mask the accessibility of the extracellular domain of the EGFR to all ligands. Conversely, it could form a heterodimer with EGFR monomers and block their dimerization. Dimerization is a prerequisite to the activation of downstream signaling events by the RTK. Alternatively, Kek1 could be involved in bringing a transmembrane tyrosine phosphatase to the vicinity of the EGFR and thus lead to its deactivation. Consistent with these models, we have observed that Kek1 can inhibit mammalian EGFR molecules from becoming tyrosine phosphorylated in response to growth factor treatment in infected insect cells (L. T. A. et al., unpublished observations). Finally, we envision that Kek1 could target the EGFR to a degradation pathway through endocytosis or bind to additional proteins involved in their subcellular localization. Studies on the *C. elegans* LET-23 EGFR have well illustrated the critical role of subcellular localization and PDZ proteins in signaling by this RTK (Kim, 1997; Kaech et al., 1998). Interestingly, we note that both Kek1 and the EGFR contain a TXV motif at the C terminus. This S/TXV motif has been implicated in protein-protein interactions and suggests that Kek1 and/or the EGFR may interact with PDZ-containing proteins (Songyang et al., 1997; see reviews by Kim, 1997 and Ponting et al., 1997). Consistent with this, our analysis of the Kek1 molecule has led us to conclude that Kek1^{extra} is less stable than the full Kek1 molecule or that the cytoplasmic domain of Kek1 participates to some extent in the inhibition. Possibly, Kek1 is targeted to a subcellular region within the cells where the EGFR itself is located. This model would explain why unlocalized Kek1^{extra} would be less efficient at inhibiting EGFR activity than the full-length Kek1 molecule.

An Emerging Theme in Patterning Mechanisms

Kek1 identifies a novel member of a growing class of negative regulators that are positively transcriptionally regulated by the pathway that they inhibit. As discussed in the Introduction, the secreted molecule Aos acts in a feedback loop to downregulate in a paracrine fashion the activity of the EGFR, possibly by preventing the mechanism of activation of the receptor by the Spi ligand (Schweitzer et al., 1995b; Golembo et al., 1996b). Another inhibitor of an RTK pathway is Sprouty, which antagonizes the activity of the FGF receptor Breathless (Btl) and is transcriptionally controlled by activated Btl (Hacohen et al., 1998).

It is worth noting, however, that not all secreted negative regulators of specific signaling pathways work in negative feedback loops. These include Chordin/SOG (Piccolo et al., 1997) and Noggin (Zimmerman et al., 1996), which antagonize signaling by TGF- β family members; FrzB proteins, which encode secreted proteins with sequence homologies to the extracellular cysteine-rich domain of Frizzled transmembrane receptors, act

as inhibitors of secreted Wnt ligands (Leyns et al., 1997; Wang et al., 1997). Finally, not all negative regulators that act in feedback loops act extracellularly. For example, Smad7, which is expressed in response to TGF- β signaling, blocks the activity of this pathway intracellularly by binding to the TGF- β receptor and by inhibiting Smad2 and Smad3 phosphorylation that is required for transduction of the signal (Nakao et al., 1997). Another example is the human CL100 phosphatase that after being expressed following exposure to either oxidative stress or heat shock downregulates specifically the MAP kinase (Keyse and Emslie, 1992). The *Drosophila* homolog of CL100, known as *puckered*, has been shown to mediate a feedback loop regulating JNK activity during dorsal closure in *Drosophila* (Martin-Blanco et al., 1998).

Kek1 Defines a Novel Gene Family

In *Drosophila*, two other putative Kek molecules that share extensive homologies with *kek1* have been identified: *kek2* (Musacchio and Perrimon, 1996) and *kek3* (our unpublished data). The function of these additional Kek-like proteins is not known. However, it is interesting to note that despite extensive saturation of the region containing *kek3*, no mutant alleles have been recovered (Ashburner et al., 1990; Spradling et al., 1995; our unpublished data). Whether or not this and the subtle effect of loss of Kek1 activity is due to redundancy within the Kekk family remains to be determined. Thus, it will be important to characterize the expression patterns as well as the loss-of-function and overexpression phenotypes of *kek2* and *kek3* in order to evaluate any potential abilities to modulate the EGFR activity. Further, chimeric proteins between these molecules may help to further define the Kek1 domain(s) required for the inhibitory mechanism.

Finally, we note that there are putative transmembrane proteins in vertebrates (Suzuki et al., 1996) and invertebrates that show similar arrangements of LRRs and Ig motifs. This raises the possibility that Kek1 is a member of a family of structurally related EGFR inhibitors. As alteration in the activity of the various members of the human EGFR/ErbB family has strong links to oncogenesis, it will be important to determine if vertebrate LRR/Ig molecules share functional, in addition to sequence, similarities to Kek1. We anticipate that the continued characterization of the Kek and related molecules will provide novel approaches to the design of inhibitors of the EGFR/ErbB family for therapeutic use in oncogenesis.

Experimental Procedures

Fly Stocks

Various collections of enhancer trap lines were screened for expression patterns within the follicular epithelium (Perrimon et al., 1991; Smith et al., 1993; D. Eberl, unpublished). From this screen, the second chromosomal enhancer trap line 15A6 was identified. For a description of 15A6, which we refer to as *kek-lacZ*, see Musacchio and Perrimon (1996). The two overlapping deficiencies, *RA5* and *RM2*, completely delete the *kek1* gene (Musacchio and Perrimon, 1996), and we refer to *RA5/RM2* females as *kek1* mutant females in this paper.

The alleles of the *egfr*, *egfr^{QY1}*, *egfr^{CJ}*, *egfr¹⁰¹*, *egfr^{2E07}* are described in Clifford and Schupbach (1989). For a description of *grk^{HK36}*, see Schupbach (1987) and Neuman-Silberberg and Schupbach (1993).

The hypomorphic *Draf* mutation *Draf^{FM7}* is described in Melnick et al. (1993). *hs-Draf^{FM7}* that expresses an N-terminal truncation of *Draf* is described in Brand and Perrimon (1994). The *UAS-EGFRDN* is described in Freeman (1996). The enhancer trap line *es(3)79*, the *P[grk]*, and the *UAS- λ top* (Queenan et al., 1997) flies were a gift from T. Schupbach.

Ectopic expression in the follicular epithelium was performed with the pGawB GAL4 lines T155 (Harrison et al., 1995) and CY2 (Queenan et al., 1997).

Inhibitor Studies

The colchicine inhibitor was used at 25 or 50 μ g/ml to destabilize microtubules as described in Theurkauf et al. (1993). As a control, flies were treated with 50 μ g/ml of β -lumicolchicine, which is an inactive isomer of colchicine. After initiating treatment, flies were dissected and fixed at 4, 8, 10, and 24 hr intervals over 48 hr and probed for expression of *grk* mRNAs. Stage 10 chambers treated after the dorsal-anterior migration of the oocyte nucleus showed a higher frequency of disrupted and irregular *kek-lacZ* expression (49/156) than chambers treated prior to the migration (17/108). Earlier treated chambers had a higher frequency of posteriorly shifted gradients (88/108 early versus 31/156 late). Finally, we detected no effects with cytochalasin D, an inhibitor of microfilaments.

In Situ Hybridization and β -Galactosidase Histochemistry

In situ hybridization was performed with digoxigenin-labeled *grk*, *kek1*, and *twist* RNA essentially as described in Tautz and Pfeifle (1989) using 55°C and 65°C as the hybridization temperature.

For detection of β -galactosidase activity, ovaries were dissected in PBT (PBS + 0.1% Tween 20) and fixed in 4% methanol-free formaldehyde for 15 min or in 2.5% glutaraldehyde for 2.5 min. Ovaries were then stained with 1 mg/ml X-Gal in X-Gal staining buffer at 25°C for 3.5–4.5 hr.

The polyclonal rabbit anti-Twist antibody (Roth et al., 1989) was used at a dilution of 1:5000.

For visualization, embryos and egg chambers were directly mounted in 80% glycerol and photographed under Nomarski optics with a Zeiss Axiophot microscope. Embryonic cuticles and chorions were prepared according to van der Meer (1977) and visualized using dark-field optics.

Constructs

UAS-kek1 was made in the following manner. A HindIII/EcoRI fragment from the *kek1* cDNA containing the complete coding region (Musacchio and Perrimon, 1996) was subcloned into pBSK⁺. From this subclone, an XbaI/XhoI fragment containing the complete *kek1* coding region was cloned into the P element vector pUAST (Brand and Perrimon, 1993). *UAS-kek1^{extra}* was made by inserting by PCR a stop codon immediately after the transmembrane domain of *kek1* and *UAS kek1^{intra}* by fusing a v-Src myristylation site to the entire intracellular domain of *kek1*. P element-mediated transformation was performed following injection into the delta 2–3 transposase strain (Robertson et al., 1988).

Insect Cell Experiments

cDNAs encoding the *Drosophila* EGFR and myc-tagged versions at the C terminus of full-length *kek1*, *kek1^{extra}*, and *kek1^{intra}* were subcloned into the baculovirus transfer vectors pVL1392 or pVL1393 (PharMingen).

Recombinant baculoviruses were produced in Sf9 insect cells using the Bac-N-Blue transfection kit (Invitrogen) and plaque purified prior to use. For coexpression experiments, 10⁷ Sf9 cells were infected at a multiplicity of infection of 30. For the EGFR/Torso comparison experiment, cells were infected with a 1:1:28 ratio of *Drosophila* EGFR:Kek1:WT baculovirus. For the domain deletion experiment, cells were infected with a 10:20 ratio of EGFR:Kek1 virus. For both experiments, cells were infected for 48 hr and then lysed in 1 ml 20 mM HEPES/Na, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, and 1 mg/ml each of pepstatin A, aprotinin, and leupeptin. Cleared lysates were immunoprecipitated with 1 μ g anti-Myc (Ab2, NeoMarkers). Precipitates were washed three times with lysis buffer, resolved by 6% SDS-PAGE, and blotted with 1/5000

anti-EGFR or 1/5000 anti-Torso. Filters were then stripped and re-probed with 1/200 anti-Myc.

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